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**Review**

**Challenges and opportunities for monoclonal antibody therapy in veterinary oncology**

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## Abstract

Monoclonal antibodies (mAbs) have come to dominate the biologics market in human cancer therapy. Nevertheless, in veterinary medicine very few clinical trials have been initiated using this form of therapy. Some of the advantages of monoclonal antibody therapeutics over conventional drugs are high specificity, precise mode of action and long half-life which favours infrequent dosing of the antibody. Further advancement in the field of biomedical sciences has led to the production of different forms of the antibody such as single chain antibody fragment (scFv), Fab, bi-specific and drug conjugates for use in diagnostic and therapeutic purposes. In this review we describe the potential for monoclonal antibodies in veterinary oncology patients in supporting both diagnosis and therapy of cancer. We explore the technical and financial hurdles to facilitate clinical acceptance of mAbs and offer insights into novel technologies and targets that could support more rapid clinical development.

*Keywords:* Monoclonal antibodies; Veterinary oncology; Therapy; Idiotypic; Biologicals

## **Introduction**

The history of monoclonal antibody (mAb) therapy can be traced back to the start of the hybridoma technology, in 1975 (Köhler and Milstein, 1975). Fig. 1 describes the chronology of mAb development.

The greatest advances in monoclonal antibody therapeutics began when recombinant DNA technology could be applied to antibody design. This facilitated the reduction in their immunogenicity and optimisation of antibody half-life and effector functions. Despite that, mAbs have taken a long time since the early days of discovery until the current “golden phase” of cancer therapies using biologicals (Waldmann, 1991). Since then, anti-cancer mAbs – and also mAbs for other therapeutic purposes – have become some of the highest selling medicines ever (King, 2013).

The importance of monoclonal antibodies as therapeutics is increasing, and currently there is an average of four new molecules reaching the human therapeutics market every year. This class of treatment is seen as critical for the future of the pharmaceutical industry, especially in a time when the development of new drugs and the discovery of new targets is declining (Munro et al., 2011; Arrowsmith, 2012; Ecker et al., 2014).

## **Mechanisms of action**

Several mechanisms of action of the monoclonal antibodies have been identified. They are summarised in Table 1 per the molecular mechanism to which they are relevant (Glennie and Johnson, 2000; Adams and Weiner, 2005).

Many antibody functions shown in Table 1 are dependent on the Fc region of the mAb. The interaction between the Fc region of the mAb and Fc receptors (FcR) is crucial for the development of immune-mediated functions of the antibody, such as antibody-dependent cell-mediated cytotoxicity (ADCC). Complement-dependent cytotoxicity (CDC) can also be activated by interaction with the Fc fraction of the mAb. This interaction is specific, such that not all antibody isotypes interact with the same intensity with the FcR, even within the IgG subclass in a single species, for instance (Bergeron et al., 2014; Strietzel et al., 2014). Therefore, the choice of Fc region during the chimerisation process (described below) affects the effector functions of the therapeutic speciated mAb. In dogs, IgGB and IgGC are capable of driving ADCC and CDC, while IgGD is only capable of activating the complement cascade (for CDC), but not of binding to FcγRs (for ADCC or phagocytosis). The other IgG isotypes of dogs, “A” and “D” are not drivers of antibody-mediated immune responses. Human IgG1 and mouse IgG2a also bind to canine FcγRI, and are potentially useful as therapeutics in dogs (Helfand et al., 1994; Bergeron et al., 2014). Indeed, mAb 231, a murine antibody previously used against canine lymphoma (described below in detail), is reported to show antibody-mediated cytotoxicity and complement activation in dogs (Rosales et al., 1988). In cats, feline IgG1a and IgG1b bind to Fc receptors and also seem capable of mediating CDC, while IgG2 does not (Strietzel et al., 2014).

IgE-Fc receptor binding is also being explored as a potential mechanism to treat cancer. There are two views on the use of IgE antibodies against cancer: 1) IgE seems to have a role in preventing cancer; 2) IgE can be used to target FcεR-expressing mast cell tumours. As for the first approach on the use of IgE, this isotype is more effective in inducing ADCC against cancer cells, as well as inducing stronger responses (Singer and Jensen-Jarolim, 2014). The second approach takes advantage of the high expression of IgE-binding Fc

receptors (FcεR) on mast cell tumours; thus, IgE can be used as a platform for the delivery of therapies to these cells (Elders et al., 2014).

## **Monoclonal antibody therapy strategies for cancer in veterinary medicine**

### *The past of mAb therapy in veterinary medicine: mAb 231*

One of the first clinical monoclonal antibody licenced for cancer treatment, denominated mAb231, was for use in canine lymphoma, although it was later discontinued. (Jeglum, 2009; USDA, 2016). Three years before the licencing of the first cancer-targeted monoclonal antibody in Germany (Panorex), and five years before the first monoclonal antibody was licenced by the FDA for the treatment of cancer in humans (Rituximab), a monoclonal antibody received approval in the US to treat lymphoma in dogs, called mAb 231. The selection of the monoclonal antibody was carried out against tumour cells and the molecular target of this antibody was never unveiled. Immunoprecipitation using the antibody did not display any protein. The antibody was nevertheless able to bind to 73% of the formalin-fixed and paraffin-embedded lymphomas tested (Steplewski et al., 1987; Jeglum, 2009). The antibody is of the IgG2aκ subclass, with a detailed study of its structure having already been performed (Harris et al., 1997). Its mechanisms of action were reported to be due to antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) (Rosales et al., 1988) and inhibition of tumour growth (Steplewski et al., 1987).

The antibody was to be administered to dogs in remission after two four weeks' cycles of vincristine, cyclophosphamide, L-asparaginase and doxorubicin (modified VCAA) and a three-week rest. The total dose was 100mg/m<sup>2</sup>, to be divided into 5 sequential days and to be administered over two hours. If lymphoma recurred after the administration of the Mab

231, a new 4-weeks cycle of the modified VCAA was to be administered, followed by a 3 weeks' rest and a new round of antibodies (Jeglum, 1996, 2009).

The complete remission of the 215 dogs tested was 80.9%, with a mean survival time of 624.28 days for the responders (in complete remission) against 153.67 days for the non-responders. Non-responders tended to be from the group of animals to which mAb231 does not bind on immunohistochemistry (Jeglum, 1996). Anecdotally, the administration of mAb 231 showed a disproportional rate of achieving indefinite remission when compared to chemotherapeutics alone (Crow, 2008).

The drug was discontinued in 1996, apparently due to lack of demand (Crow, 2008; Kaplan, 2008). This mAb is no longer licenced for use by the USDA (USDA, 2016). Yet, it must be brought to attention that the drug was marketed in a time when antibody therapy to cancer was non-existent in human medicine, nor had the subject the same attention it receives currently (Reichert, 2012).

#### *The present of mAb therapy in veterinary medicine: antibodies reaching the market*

The veterinary market is currently going through its first intense wave of interest in monoclonal antibodies (Harvey, 2015; Ledford, 2016). Two product launches by Aratana Therapeutics, against B (product AT-004, anti-CD20) and T cell lymphomas (AT-005, anti-CD52) were widely regarded in the market as great promises for veterinary oncology. AT-004 was shown to increase median progression-free survival of dogs with B cell lymphoma (Anderson and Modiano, 2015). However, recent post-USDA licencing trials have not shown encouraging results; in randomized clinical trials combining AT-005 to two different chemotherapy protocols, mAb therapy did not seem to improve clinical outcomes compared

to placebo. Aratana indicated that both AT-004 and AT-005 showed poor specificity to their respective targets. Despite this, both antibodies are currently still commercially available<sup>1</sup>.

While other commercially available options of mAbs for the treatment of cancer in animals are not yet available, there are candidates approaching or at market launch for other clinical conditions. Zoetis has recently been granted a conditional licence by the USDA for lokivetmab, to be used for treatment of canine atopic dermatitis. This mAb blocks IL-31, which is important for the development of pruritus (Michels et al., 2016), with the intent of interrupting the cycle of itching and inflammation typical of atopic dermatitis (Zoetis, 2015)<sup>2</sup>. Nexvet Biopharma is investigating a target for chronic pain, with an anti-nerve growth factor mAb. Interestingly, they have already demonstrated the efficacy of this mAb both in caninised and in felinised formats (Gearing et al., 2013, 2016)<sup>3</sup>.

#### *The future of mAb therapy in veterinary medicine I: possible targets*

There is a limited number of publications evaluating the effects of monoclonal antibodies or related molecules in veterinary species. Shown below in Table 2 are antibodies that have been trialled or used in domestic animals, as well as future potential targets for these patients.

#### *The future of mAb therapy in veterinary medicine II: 'speciation'*

Veterinary medicine has always borrowed tools from human practice. However, contrarily to chemical drugs, biologicals such as mAbs are in most cases species-specific.

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<sup>1</sup> See: <http://aratana.investorroom.com/2015-09-24-Aratana-Therapeutics-Provides-Product-Updates> (accessed 15 June 2016).

<sup>2</sup> See: <http://news.zoetis.com/press-release/companion-animals/zoetis-receives-conditional-license-usda-canine-atopic-dermatitis-im> (accessed 16 June 2016)

<sup>3</sup> See: <http://ir.nexvet.com/phoenix.zhtml?c=253841&p=irol-newsArticle&ID=2176442> (accessed 16 June 2016).



Although the process of antibody caninisation is now becoming more widely used, it is still a time-consuming method (Gearing et al., 2013). Also, producing mAbs exclusively for one animal species may not be always commercially viable, and may impede further developments of new candidates. This is clearly illustrated by the fact that while there are now tens of monoclonal antibodies approved worldwide for cancer therapy in humans (Modjtahedi et al., 2012; Reichert, 2012; Ecker et al., 2015), in veterinary oncology, however, therapeutic antibodies are only just reaching the market (USDA, 2016).

The use of antibodies that have been developed for human therapy in animals is an attractive approach. Some groups have explored the potential use of human mAbs in dogs. Stein and colleagues, for instance, successfully tested an anti-human leukocyte antigen DR in dogs. While it was then concluded that dogs were validated as good models for the trial of an antibody, the authors also note how the study and the patients would have benefited from a species-specific antibody (Impellizeri et al., 2006; Stein et al., 2011; Biller et al., 2014).

Monoclonal antibody 231, the best example of mAb therapies in veterinary medicine, may have had reduced efficacy due to its xenogeneic origin. The canine anti-mouse antibody (CAMA) levels were raised after therapy with this antibody, and, as expected, further applications resulted in quicker and higher production of CAMA. No clinical disease seemed to arise from the use of this murine antibody in dogs. The investigators believed that length of remissions and survival times were not affected by the murine nature of the antibodies, but it was never directly compared to a ‘caninised’ version of the antibody (Jeglum, 1996, 2009). Possible side effects were occasionally observed, including facial oedema, erythema, fever, pruritus, vomiting and/or diarrhoea, joint pain and myalgia. Although the antibody seemed to

provide clinical benefits, it is possible that its murine origin was part of the reasons why it was later discontinued (Jeglum, 1996, 2009).

Much more likely to be successful is the interchange of therapeutic antibodies between dogs and cats, for instance. Fc receptors, which are crucial for antibody half-life and effector functions, are very similar between these two domestic species (tested using Ensembl Genome Browser and ClustalOmega<sup>4</sup>) (Lobo et al., 2004; Strietzel et al., 2014). This could prove to be an important feature for veterinary practitioners when new mAbs become available for either species, since development of mAbs for these patients is slow and receives relatively little attention, especially for cats, for whom no mAb is available currently in the market.

Even though the majority of the literature indicates that anti-globulin responses limit the function of mAbs, as discussed above, there are still licensed antibodies for use in humans that are not humanised. Understanding how they are effective may set new parameters that may be useful for the production of innovative antibodies for domestic animals.

At least two mouse antibodies are still currently in use for the treatment of malignancies in humans. Both of these target CD20, and both are conjugated to radionuclides. The use of murine antibodies in humans is permitted in these cases because the mAbs are clinically useful with single dose schemes. Rituximab, a chimeric mAb that also targets CD20 in humans, is used in a course of several doses, in comparison (Witzig et al., 2002; Burdick and Macklis, 2009). Further possible explanations for the low seroconversion from the use of these antibodies include the occurrence of immune suppression. It is expected

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<sup>4</sup> See: <http://www.ensembl.org/index.html> and <http://www.ebi.ac.uk/Tools/msa/clustalo/> (accessed 6 March 2015).

that both normal and malignant B cells are depleted by the treatment, therefore limiting the capacity of the host to react to the mAbs (Mirick et al., 2004). Therefore, these results leave open the possibility of using non-specified mAbs in animals if these are administered in single-dose regimens; mAbs coupled to radionuclides are potential candidates.

### **The process of speciation**

As should be expected, xenogeneic antibodies are more immunogenic than syngeneic ones. Therefore, speciation was probably the single most important step towards the success of mAbs in clinical practice, since anti-mAb responses are responsible for rapid clearance of circulating mAbs as well as for several side effects (Glennie and Johnson, 2000). Chimerisation was the first stage towards antibody speciation (Hwang and Foote, 2005). In this technique, after the antibody is raised and selected from mouse hybridomas, the murine variable regions are cloned with human (or from any other species of interest) constant regions by fusing the immunoglobulin genes in an expression vector (Fig. 2). This is then expressed in a host system, such as Chinese hamster ovary (CHO) cells (Köhler and Milstein, 1975; Persic et al., 1997).

Humanisation is a further step in the engineering of the antibodies. This technology starts from the chimerised version of the mAb, and attempts to further reduce the immunogenicity of the antibody. In speciation, the framework regions of the variable fraction (the parts of the variable fraction which do not contact the antigen) of murine antibodies are substituted with human germline sequences. In this approach of speciation, CDRs are retained from the original murine mAb sequence.

To completely avoid murine sequences, additional strategies have been developed to generate fully speciated monoclonal antibodies through display technologies (such as phage display) and transgenic mice (Deckert, 2009; Bradbury et al., 2011). In fully human antibodies, all of the mAb molecule, including the CDRs, which bind to the antigen, are from the species of interest (Thie et al., 2008). Phage display relies on libraries of antibody fragments or whole IgGs (reviewed by Miersch & Sidhu, 2012) (Fig. 3). These can be constructed from samples of the species of interest; thus, a display library can contain the virtually the entirety of the antibodies being expressed in a blood sample, or a spleen, for instance. The ultimate aim of the display technology is the selection of the recombinant antibody that can bind to the antigen with highest affinity from the immense number of non-specific antibodies in the library. After the construction of a display library putatively containing all the possible antibodies from one animal – or from several animals, if desired – this library can be screened against different therapeutic targets by the process of biopanning (Barbas et al., 1991). Since the library can be constructed from the target species, instead of mice, the antibodies it generates will be fully human (or canine, for example). Mice modified to contain a ‘human’ immune system are also used as an alternative for the production of fully human mAbs, but such a technology is not available for production of antibodies for domestic animals (Shultz et al., 2012). The aforementioned technologies associated with antibody production are illustrated in Fig. 4.

Although full speciation of antibodies has become the industry standard (Modjtahedi et al., 2012), more recently opinions have emerged questioning the real benefits of engineering antibodies beyond chimerisation. As stated previously, chimerisation is responsible for the main reduction of immunogenicity of the mAbs. Indeed, in many cases chimerisation is enough to prevent anti-mAb formation. Full speciation also has the

disadvantages that it is a lengthy and labour-intensive laboratory process, delaying product launch, and in many cases leads to lower antibody affinity, since it interferes with the antibody variable regions (Kettleborough et al., 1991). New speciation techniques have been developed to try and circumvent these problems; one such example is the “PETization platform”, developed by a veterinary company (Gearing et al., 2013). However, full speciation does not guarantee that a mAb will not raise a host response, as has been seen with some fully humanised mAbs (Clark, 2000; Singh et al., 2012; van Meer et al., 2013; Waldmann, 2014).

In veterinary medicine, the first speciated mAbs are now attaining the necessary legal approvals and are entering the market (Acharya et al., 2015), and it will be interesting to analyse the responses of large cohorts of animals to such caninised antibodies, in special with regards to side effects.

### **Potential side effects of mAb therapy**

The side-effects of mAb therapy can arise as a consequence of a host reaction against the antibody or of the direct activity of the mAb. The host reactions against mAbs can be either acute (infusion reactions or anaphylactoid) or dependent on the formation of anti-mAbs. Acute reactions include the cytokine release syndrome (CRS) and systemic inflammatory response syndrome (SIRS). A number of counter-measures are recommended, such as hydration, premedication and gradual increase in the rate of infusion. Anaphylactic and ‘anaphylactoid’ reactions occur more frequently with some antibodies than with others, probably due to the generation of IgE by the host. Adverse reactions can also be derived from the formation of anti-mAb antibodies after administration. These tend to appear and increase with subsequent doses, when the immune response has matured to secondary and adaptive

reactions. In these cases, high titres of anti-mAb IgGs can be found in the circulation of the patient. Monitoring anti-mAb responses relies on specialized techniques, not commonly available to the veterinary clinician; it depends on the concomitant development of the mAb with its coupled monitoring tests. Anti-mAb responses can be monitored, among other possibilities, by specific enzyme-linked immune sorbent assays (ELISAs), such as anti-idiotypic ELISA (for an explanation on anti-idiotypes, see Fig. 5 below and associated discussion) or antigen capture methods (Shankar et al., 2007; Renaudineau et al., 2009).

Anti-mAb reactions may cause CRS and influenza-like symptoms in humans (McLaughlin et al., 1998; Hansel et al., 2010). In dogs, the murine mAb 231 caused clinical signs such as oedema and fever, but the occurrence of these signs did not impede subsequent administration of the treatment; however, the dogs were preventively treated with antihistamine, corticosteroids and the rate of infusion was reduced (Jeglum, 1996). It must be noted that side effect management and mAb treatment withdrawal should be considered on a case-by-case basis (Melosky et al., 2009). These host reactions tend to be more frequent when non-speciated (humanised or caninised) formulations are used, demonstrating the relevance of speciation (Adams and Weiner, 2005). The speciation process of antibodies, from chimerisation to full speciation, followed by fully human (or canine, feline) mAbs, reduces but does not exclude the probability of formation of strong host adverse reactions against the antibody. Moderate and mild host reactions may continue to occur, nonetheless (Hwang and Foote, 2005).

Reactions against fully speciated mAbs (canine against canine antibodies, for instance) occur due to the inherent variability of antibodies. The antigen-binding region (named the complementarity-determining region, or CDR) is by definition always structurally

novel to the immune system itself – that means that antibodies are always immunogenic, regardless of how speciated the constant region of the mAb is. Although it is possible to change a few (1-2) amino acids in the CDR of a mAb to reduce its immunogenicity, these alterations always risk reducing the functionality of the antibody, since its very binding region is being altered (Harding et al., 2010). The immunogenic property of the CDRs create the phenomenon of the idiotype cascade presented in Fig. 5 (Herlyn et al., 1986). The main importance of full speciation seems to be to reduce “marked” anti-antibody responses. When reactions do occur against speciated mAbs, these tend to be “tolerable” or “negligible” (Hwang and Foote, 2005).

The most common signs that follow the rapid infusion of rituximab, a human chimeric antibody, are vomiting and nausea, rash and hypotension. The prophylactic use of antipyretics and antihistamines is widespread to prevent these effects (Lang et al., 2011). However, major acute reactions such as CRS can occur. Similar effects are also known to occur after the administration of other chimeric mAbs such as Cetuximab (an anti-EGFR mAb). These strong adverse reactions are less common for fully human mAbs, such as Panitumumab (also anti-EGFR), but signs, such as skin rashes, still occur widely with the use of this antibody (Hansel et al., 2010). Nevertheless, it has been highlighted that the most common biological consequence of anti-mAb responses tends to be loss of efficacy, which is deleterious but not a safety concern (Shankar et al., 2007).

The second type of possible reactions is dependent on antibody activity. One possible effect of mAbs is the formation of Tumour Lysis Syndrome (TLS), where the release of intracellular contents from the tumour after being targeted by treatment leads to metabolic disorders, such as hyperkalaemia, hyperphosphatemia and hypocalcaemia (Jeha, 2001;

Gilbert and Wright, 2015). Undesired antibody activity has led to life threatening situations in the famous case of the CD28 superagonist antibody TGN1412, used with the goal of stimulating immune responses. This mAb had been trialled in human cells *in vitro*, in mice and in primates before reaching a human phase I clinical trial. However, in humans the mAb induced a severe cytokine storm (CRS): within minutes of administration patients had headaches, nausea, vomiting, pyrexia, a systemic inflammatory response with hypotension. This was followed by severe respiratory deterioration and several other signs of metabolic failure. It was later found that these effects were not predicted by pre-clinical tests due to immune differences between primates and mice compared humans – CD4 from macaques lose CD28 expression at the effector memory level, while mice induce strong regulatory T lymphocyte responses when administered with the superagonist. Additionally, the mere detail of cell density in cell culture assays seems to have affected the results so that the cytokine storm was also not predicted from these tests (Suntharalingam et al., 2006; Hünig, 2012).

Other side-effects related to antibody function may derive from off-targets. None of the currently approved anti-cancer mAbs target a tumour-specific molecule. The targets are also expressed in normal tissues, albeit often at different concentrations. Adverse reactions therefore may originate from the off-targeting of the mAbs to molecules associated with physiological functions, but these events are rarely life threatening or debilitating (Modjtahedi et al., 2012).

When an antibody is used to block the function of leukocytic cancers, the possibility of side effects related to suppression of normal immune cells exists. Alemtuzumab, used for lymphoid malignancies, targets CD52, which also exists on the surface of normal circulating lymphocytes, leading to lymphocytopenia for up to one year after treatment is discontinued.



One of the possible consequences is the appearance of opportunistic infections. Rituximab targets CD20 on the surface of B cells, but secondary infection is normally only a problem in patients that were already immunosuppressed or taking immunosuppressive drugs concomitantly. Nevertheless, transient decline in leukocytes can occur after use of Rituximab and caution is recommended when administering multiple courses of the antibody. Additionally, reactivation of latent hepatitis B infections has been recorded in patients receiving Rituximab (Sharma et al., 2007; Kelesidis et al., 2011; Uettwiller et al., 2011).

### *Benefits of immunosuppression*

Several factors influence the formation of anti-antibody responses by the patient receiving a mAb, such as the number of injections of the antibody, its immunogenicity and the immunocompetence of the recipient (Kuus-Reichel et al., 1994). Reducing the immune response against the mAb is therefore an intuitive procedure that could make possible the use of extraneous antibodies or reduce the responses against antibodies. Indeed, it is believed that the lack of a negative reaction to some antibodies is due to the immunosuppressed status of the patients, either by the use of chemotherapy or because of the cancer activity itself (Glennie and Johnson, 2000). Suppression of human anti-mouse (HAMA) responses has been attempted with the administration of cyclosporine and other compounds. The use of cyclosporine was able to reduce the formation of anti-mAb responses and increase the amount of useful doses of mAbs that could be administered, but could not completely suppress HAMA in all the individuals tested or for an indefinite amount of administered doses (Ledermann et al., 1988; Weiden et al., 1994; Dhingra et al., 1995). CTLA-4 inhibits T lymphocyte activation by affecting the CD28-B7 co-stimulatory signal. Healthy beagle dogs simultaneously receiving an antibody fraction and the recombinant human CTLA-4 had better antibody responses. While dogs treated with the CTLA-4 had no side-effects after 5

doses of human antibody fractions, dogs that did not receive CTLA-4 simultaneously with the antibody showed hypersensitivity responses after 4 doses (pale mucous membranes, tremors, nasal discharge, and other signs). Also, the treatment seemed to reduce anti-antibody formation, allowing antibody plasma levels to be higher for longer periods (Siegall et al., 1997). However, another study could not reproduce these results. The addition of CTLA-4 did not inhibit the formation of anti- antibodies in this second study (Henry et al., 2005).

### **Hurdles and opportunities**

The hurdles and opportunities in the study and production of new antibodies for veterinary use are discussed below and are summarised in Table 3.

The immunogenicity of therapeutic molecules, including monoclonal antibodies, affects the efficacy and safety of the product (Chirino et al., 2004). For the veterinary field this still represents the greatest barrier for mAb usage in the clinical setting, although much still needs to be studied about the possible use of non-speciated mAbs. Clinical use of antibodies will benefit from the production of species-specific molecules (Gearing et al., 2013).

Cost represents an important concern for monoclonal therapies in humans. In veterinary medicine, this issue is even more relevant. The need for high cumulative doses and the fact that cancer is a chronic disease add to the fact that current methods for antibody production in mammalian cells are intrinsically expensive. Novel technologies such as transgenic animals and use of yeast, bacteria and insect culture systems are probable paths to be followed in the future for the production of antibodies (Daniell et al., 2001; Farid, 2007). This highlights the importance of better understanding the possibilities of using single-dose

non-speciated mAbs, which are substantially cheaper to produce, since the price of production will likely hinder the market accessibility of the veterinary biologics industry.

Another limiting factor is the ability of the antibody to penetrate into the tumour. This accounts for one of the reasons why many mAbs that are promising during the developmental stage are not so successful clinically. Clearance from circulation, blood vessel density, vascular permeability and binding site barrier are some of the factors that influence mAb delivery (Lobo et al., 2004; Niu et al., 2009). Solutions include the use of antibody fragments such as scFv or Fab which exhibit better pharmacokinetics for penetration into the cancerous tissue and also possess high binding specificity (Batra et al., 2002). Direct intratumoural antibody administration and the administration of microencapsulated whole antibody-producing cells or other *in situ* antibody producing technologies are possible means to circumvent these difficulties (Pelegrin et al., 1998; Azemar et al., 2003; Orive et al., 2003). Imaging techniques such as positron emission tomography (PET) offer high sensitivity which is required to monitor distribution of the drug throughout the body, to study pharmacokinetics and pharmacodynamics and also provide the information on optimal timing and antibody dosage (Rudin et al., 2005). These techniques will be central in the establishment of better mAb tumour penetration.

Reduced efficiency of monoclonal antibodies during human clinical trials when compared to mouse trials is of major concern (Stein, 1997), and this might also be an issue when mAbs are to be used in veterinary medicine. Dogs represent a good model to study cancer on in respect to possible future applications in human patients. The genetic diversity of dogs is comparable to that of humans, and is larger than that of most rodent models; their cancer-associated genes show great similarities; cancers are naturally occurring, and appear

in animals with intact immune systems; similar factors influence the appearance of cancer in dogs and humans, such as age and environmental exposure to carcinogens. Indeed, clinical studies in dogs have shown that they serve as good predictors of both clinical toxicity and response to therapy (Paoloni and Khanna, 2008; London et al., 2009; Richards and Suter, 2015). It would be in the interests of both veterinary medicine and of human medicine that dogs became an established intermediate in the studies of monoclonal antibodies. Experiments using human or murine mAbs in animals need to consider the potential benefits of the idiotype responses and the possibility of using mAbs in short dosing protocols. Quick chimerisation of antibodies and the study of cheaper expression systems would facilitate this process. It is very likely that once an antibody is fully arranged to be tested in dogs, the relevance of the results for human clinical trials would be improved when compared to mouse studies.

## **Conclusions**

The current trend of biological therapeutics is now reaching veterinary medicine. While there are still many challenges to overcome in order to achieve commercial viability and wide accessibility of monoclonal antibodies for animal use, there are many opportunities in this field, as can be seen by the renewed interest of many research laboratories and private companies. Testing and using monoclonals clinically in veterinary medicine will be of benefit for both the animal patients and the development and improvement of human therapeutics.

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## Conflict of Interest

The authors declare no conflicts of interest.

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887 **Table 1**

888 Molecular mechanisms of action of monoclonal antibodies in cancer.

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Classes	Mechanisms	Mediators	mAbs	Effects
Direct effects				
Blockade of signalling	Neutralisation of mediators, cytokines, blockade of receptors, induction of apoptosis	Apoptosis mediators	Daclizumab	Inhibition of proliferation and apoptosis
			Trastuzumab (other mechanisms also involved)	
			Cetuximab	
			Panitumumab	
			Nivolumab	
Induction of signalling	Protein tyrosine phosphorylation, upregulation of Myc	p38 Map kinase, protein kinase C, Myc	Ipilimumab	Regulation of cell growth
			[nearly all commercially available antibodies for cancer affect signalling to some extent]	
Conjugated antibodies	Induction of cell death and inhibition of proliferation	Drugs, toxin fragments, radioisotopes	Tositumomab	Direct cell death
			Ibritumomab tiuxetan	
Immune Mediated Effects				
ADCC <sup>a</sup>	Interaction of mAb with FcγR bearing cells (NK, macrophages, neutrophils) with mAbs of IgG2a and IgG3 (in dogs receiving mouse mAb) or IgG1 and IgG3 (in humans)*	[NK cells] IFNγ, cytotoxic granules (perforin, granzymes), [Macrophages] ROS, proteases	Rituximab Trastuzumab (other mechanisms also involved) Alemtuzumab	Inhibition of cell proliferation, anti-angiogenesis, cell lysis. The antigen presentation following cell death may induce a host

				response.
<b>CDC</b>	Classical activation of complement system	IgG1 and IgG3 Fc region (in humans) <sup>b</sup> ; IgG2 region (in mice) and complement cascade (initiating with C1q)	Alemtuzumab Rituximab	Lysis of cells by the complement through the creation of pore on cell membrane. Enhancement of ADCC.
<b>Immunomodulation</b>	Blockade of immune inhibitory receptors; approximation of immune cells to its target (trifunctional antibodies); activation of cells and induction of immune memory	CTLA-4, PD-1, CD40, CD3, Fcγ receptors (mouse IgG2a and rat IgG2b in combination, for humans) <sup>c</sup>	Catumaxomab; Ipilimumab	Induction of T mediated lysis, ADCC, phagocytosis; reduction of tumour-induced immune anergy
<b>Checkpoint inhibitors</b>				

<sup>a</sup>ADCC, antibody-dependent cell-mediated cytotoxicity; CDC, complement-dependent cytotoxicity; IFN, interferon; FcγR, Fc gamma receptor; ROS, reactive oxygen species; NK, natural killer cells.

<sup>b</sup>Shuffled (engineered) isotypes of IgG1 and IgG3 in humans show a strong enhancement in the ADCC and CDC capacity (Kubota et al., 2009)

<sup>c</sup>Shuffled isotypes of mouse IgG2a and rat IgG2b show increased activation of human FcγR.

References: (Rosales et al., 1988; Helfand et al., 1994; Pedersen et al., 2002; Iannello and Ahmad, 2005; Seimetz et al., 2010; Eissler et al., 2012; Golay and Introna, 2012; Anderson and Modiano, 2015) .

908

909 **Table 2**

910 Potential targets in domestic animal species for monoclonal antibodies in oncology.

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Target (name of mAb)	Condition targeted	Potential to be used in animals	Outcome in dogs (cats)	Hurdles	References
<b>CD20</b>	B-cell lymphoma	Dog PBMC stained with Rituximab. CD20 occurs in canine B-cell lymphomas. New antibodies developed for dogs (AT-004, 6C8, 1E4)  mAb 6C8 had specific binding to canine B-cells  mAb 1E4 (Elanco) has 'rituximab-like' properties and depletes B-cells; chimeric version maintains function	Rituximab does not bind peripheral blood dog cells. mAbs AT-004 reached the market [Aratana/Novartis], with poor outcomes.	Difference in aa <sup>a</sup> where Rituximab binds.  Low affinity of AT-004 for its target	(Jubala et al., 2005; Binder et al., 2006; Impellizeri et al., 2006; Acharya et al., 2015; Aratana Therapeutics Inc, 2015; Ito et al., 2015; Rue et al., 2015)
<b>CD52</b>	T-cell lymphoma	mAb received USDA approval for therapeutic use in dogs (AT-005)	Canine mAbs reached the market, but with poor responses [Aratana]	Low affinity of the antibody for its target	(Acharya et al., 2015; Aratana Therapeutics Inc, 2015)
<b>CD47</b>	B-cell lymphoma	Innate anti-tumor immune response such as phagocytosis increased against canine lymphoma by anti-CD47 mAb	mAb treatment increased canine lymphoma phagocytosis <i>in vitro</i>	None described	(Anderson and Modiano, 2015)(Anderson et al., 2016)
<b><math>\alpha 4\beta 1</math> receptor (LLP2A)</b>	NHL	Biodistribution of LLP2A studied in dog as model for human	Tumour affected tissues strongly targeted (preferentially B cells)	None described	(A L Zwingenberger et al., 2012; Allison L Zwingenberger et al., 2012)
<b>Lymphoma [target not defined] (mAb 231)</b>	Lymphoma	FDA approved for dog lymphoma. Binds 73% of tested lymphomas	Increased complete remission in dogs combined with VCAA*	Discontinued due to lack of demand. No longer USDA licensed	(Steplewski et al., 1987; Jeglum, 1996, 2009; USDA, 2016)
<b>VEGF (Bevacizumab)</b>	Various cancers	VEGF correlation with outcome ambiguous in dogs and cats. Molecular structure of VEGF/R similar between dog and man.  Bavacizumab inhibited canine osteosarcoma and	Delayed tumour growth and blood vessel formation in mice grafted with canine osteosarcoma	Humanized mAb and cost of therapy	(Scheidegger et al., 1999; Millanta et al., 2002, 2006; Restucci et al., 2002; Coomer, 2008; Michishita et al., 2013; Scharf et



		hemangiopericytoma xenografts in mice.			al., 2013)
<b>Lewis<sup>y</sup> (BR96)</b>	Breast and lung carcinomas	33% of dog tumour samples tested express Le <sup>y</sup> (several carcinomas)	Partial response in 25% of dogs tested. Stable disease in 33%. Dogs negative for Le <sup>y</sup> had progressive disease.	75% cases had anti-mAb reactions not preventable by immunosuppression. Anti-mAb reactions curtailed clinical benefits	(Friedman et al., 1993; Henry et al., 2005)
<b>HER2/neu (trastuzumab)</b>	Breast cancer overexpressing HER2/neu	Dog tumours expressing HER2/neu have been identified, but prognostic value is uncertain. Canine and human receptors are similar; dogs have a similar antigenic phenotype to humans. Prognostic value and tumour behaviour are more similar to humans in cats	Human monoclonal antibodies are able to bind dog antigens.	In cats no adaptation is possible from trastuzumab, since human and feline receptors differ at the EC* region	(Mottolese et al., 1994; Demaria et al., 2005; Valabrega et al., 2007; Gama et al., 2008; Gianni et al., 2010; Singer et al., 2012)(Cannon, 2015)
<b>CEA*</b>	Tumours of colon; other epithelial tumours	CEA ligand and receptor aa* are 99% conserved in dogs compared to humans.	CEA receptor is expressed in canine mammary carcinoma cell lines	The CEA ligand is not differentially expressed in cancer in dogs	(Watine et al., 2001; Sturgeon et al., 2008; Weichselbaumer et al., 2011; Campos et al., 2012; Grunnet and Sorensen, 2012)
<b>EGFR (cetuximab)</b>	Colorectal; head and neck cancer	In canine mammary tumours, EGFR has been associated with malignancy and poor prognosis. Human and canine receptors are similar in the epitopes for cetuximab. A caninised version of Cetuximab was developed	Cetuximab is tumourstatic to canine mammary carcinoma cell lines.  Caninised cetuximab (can225IgG) maintained function	Cetuximab is a human chimeric antibody.  Caninised version is in trial phase.	(Gama et al., 2009; Shiomitsu et al., 2009; Selvarajah et al., 2012; Singer et al., 2012, 2014; Terragni et al., 2014; Acharya et al., 2015; Queiroga et al., 2015)
<b>Human leucocyte antigen-DR [MHC II] (Murine version: L243; humanised: IMMU-114)</b>	Lymphoma	L243 and IMMU-114 bind human lymphocytes and malignant cells in nodal tissue	Transient disease stabilisation.  Murine led to fever, vomiting and short mAb half-life. Humanised had lymphocytopenia and half-life of 2h	Author recommended the canine chimerisation of mAb	(Stein et al., 2011)
<b>Prostatic tumour antigens</b>	Prostate cancer and human colon	Met5 binds to the EC region of human and canine Met; dog prostate express PSMA mRNA	Met5 binds to canine prostate cancer metastatic cell line	None described	(Anidjar et al., 2001; Ross et al., 2003; Hay et al.,

(Met5; PSM-P12; CC49)	cancer	and 50% of cancers have the antigen (but not benign tissue)	GN4; PSM-P12 binds to a prostate cell line (DPC-1); CC49 was successful in imaging canine metastases	2005; Lewis et al., 2005; Lai et al., 2008)	
PD-1	Several tumour types	Several canine tumours express PD-1, and some also express its ligand. Intra-tumoural lymphocytes in canine melanoma also express PD-1. Anti-PD-1 increases IFN $\gamma$ production	PD-1 being researched by Nexvet/Zenoaq	None described	(Maekawa et al., 2014, 2016; Nexvet, 2016)

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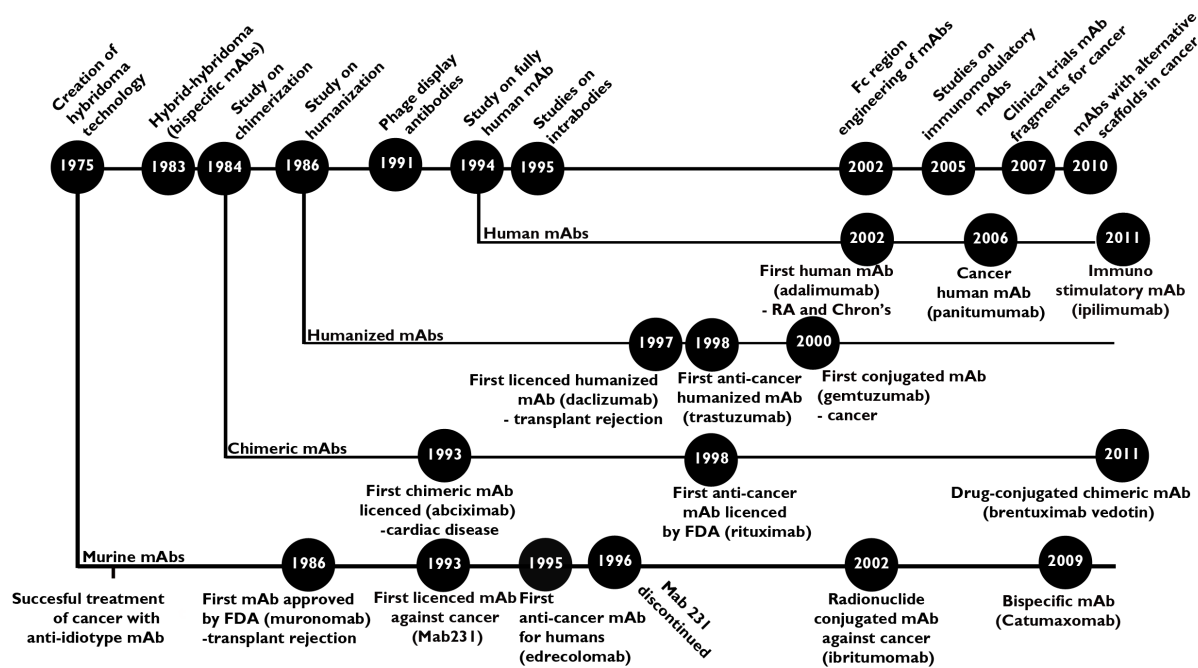
913   <sup>a</sup>aa = aminoacids; CEA = carcinoembryonic antigen; EC = extracellular; EGFR = epidermal  
914   growth factor receptor; PSMA = prostate specific membrane antigen; VCAA = vincristine,  
915   cyclophosphamide, L-asparaginase and doxorubicin; VEGF/R = Vascular endothelial growth  
916   factor/receptor;

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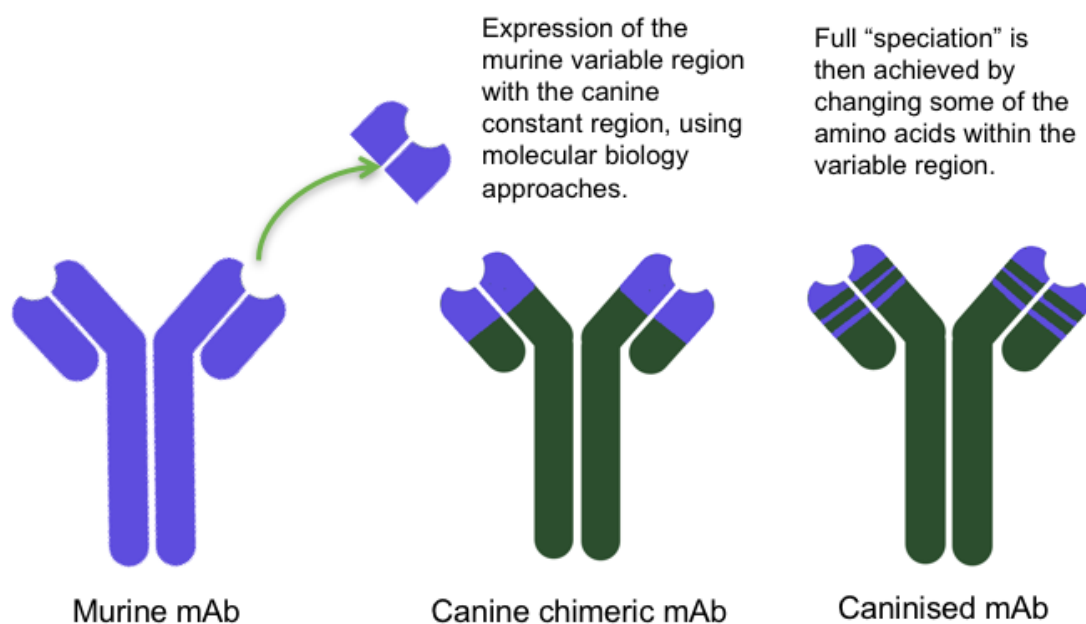
**Table 3**

Hurdles and opportunities in monoclonal antibody therapies in veterinary medicine.

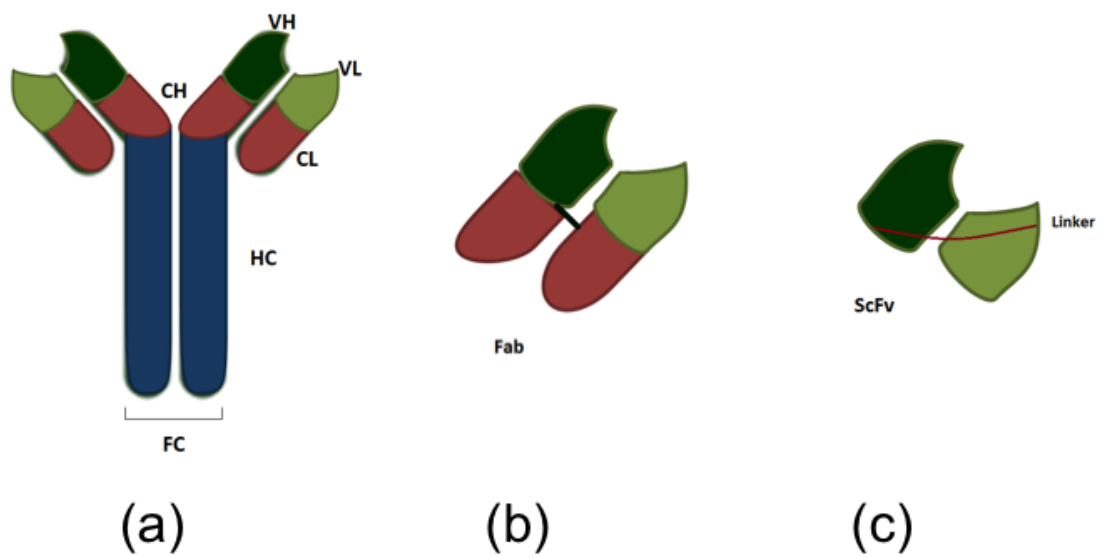
Hurdles	Opportunities
Immunogenicity of human and mouse mAbs in dogs	Caninised and chimeric mAbs
Cost of production of conventional mAbs, with cumulative doses	Alternative mAb production systems and the use of non-speciated single-dose mAbs. Possible expression systems: Insect and yeast cells, transgenic plants and animals. Bacterial production of scFv
Difficult tumour penetration	<i>In vivo</i> production of the antibody by the host, through viral transduction or direct DNA injection; use of scFv antibody fragments
Weak results <i>in vivo</i> following promising results in mice	Use of naturally occurring cancers in animals as models to study the therapies



926 Fig. 1. History of monoclonal antibodies and its clinical uses. The top horizontal line presents  
 927 the main technological breakthroughs and the year in which they occurred (or the year around  
 928 which significant research was done on that subject). The parallel lower lines represent the  
 929 mAbs used clinically categorised by their structural classes with regard to humanisation. The  
 930 mAbs cited represent a milestone in the use of the technology; other dates of interest are also  
 931 shown. RA = rheumatoid arthritis; References: Carter et al., 2001; Lobato and Rabbitts, 2001;  
 932 Fong and Small, 2008; Reichert, 2012; Kellerman and Green, 2002; Enever et al., 2009.

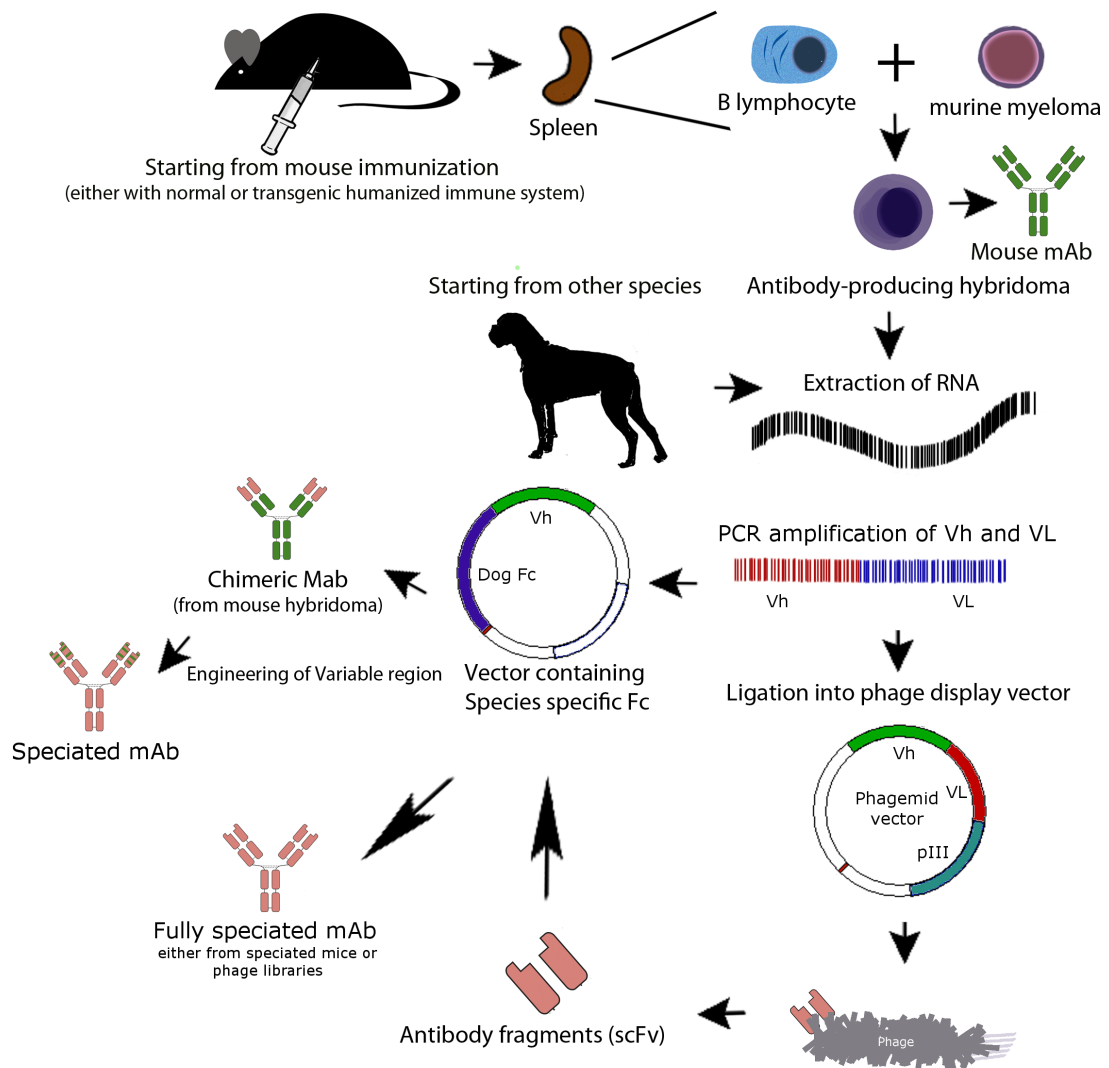


933  
 934 Fig. 2. The most common methods of antibody production and the resulting classes of  
 935 antibodies (Jakobovits et al., 2007).



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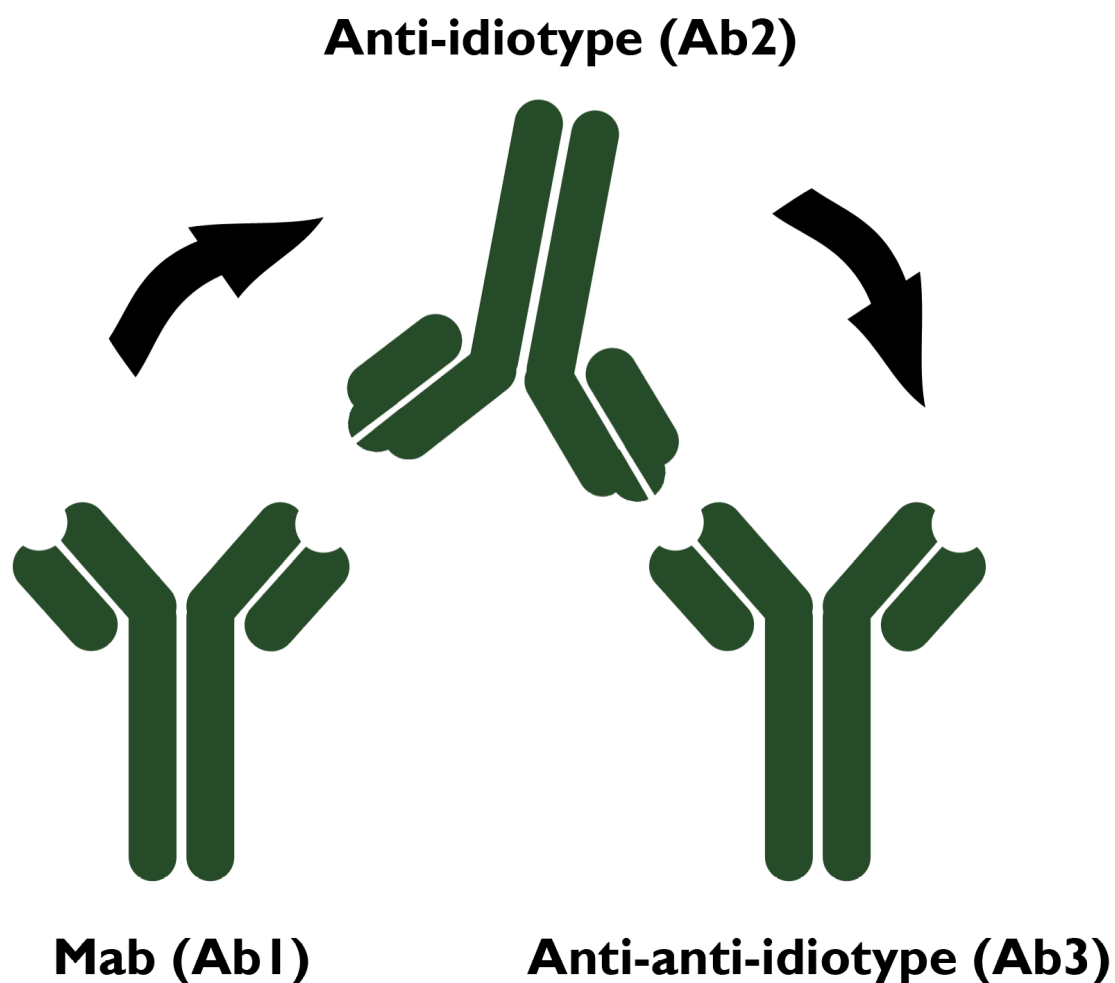
937 Fig. 3. Representation of different formats of the antibody molecule. (a) Full length  
 938 monoclonal antibody showing heavy as well as light chain comprised of variable and  
 939 constant regions, also the hinge region and the Fc portion which interacts with cell surface  
 940 receptors; (b) Fab molecule which contains no Fc portion of the antibody, thus containing  
 941 variable as well as constant regions from heavy and light chains; and (c) scFv which contains  
 942 only variable domains of the heavy and light chains for antigen binding.



943

944 Fig. 4. Techniques for producing antibodies of different speciation levels. Antibodies can be  
945 derived either from: 1) mice or other laboratory animals immunized with the antigen of

946 interest; unless the mouse was altered genetically to express the immune system from another  
947 species (such as the XenoMouse), antibodies derived from laboratory species will be at most  
948 ‘speciated’ (humanized, for instance); 2) directly from the species of interest, in which case  
949 phage display can be used to select the antibody of interest from the vast repertoire available  
950 in any animal. In this case, the antibodies that are derived are ‘humanized’, or ‘caninised’,  
951 etc.



952  
953 Fig. 5. The idiotype cascade. The mAb itself is an antigen, and the patient receiving it will  
954 raise an immune response against all the foreign parts of the mAb. Ab2 are created by the  
955 host as a response against the antigen-binding region of the mAb (Ab1). Ab3 is then raised  
956 recognizing the antigen-binding region of Ab2. Therefore, the antigen-binding region of Ab3  
957 is identical to that of the therapeutic mAb (Ab1).



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